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Original Research

PAR4 (Protease-Activated Receptor 4) Antagonism With BMS-986120 Inhibits Human Ex Vivo Thrombus Formation

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Objective—BMS-986120 is a novel first-in-class oral PAR4 (protease-activated receptor 4) antagonist with potent and selective antiplatelet effects. We sought to determine for the first time, the effect of BMS-986120 on human ex vivo thrombus formation.

Approach and Results—Forty healthy volunteers completed a phase 1 parallel-group PROBE trial (Prospective Randomized Open-Label Blinded End Point). Ex vivo platelet activation, platelet aggregation, and thrombus formation were measured at 0, 2, and 24 hours after (1) oral BMS-986120 (60 mg) or (2) oral aspirin (600 mg) followed at 18 hours with oral aspirin (600 mg) and oral clopidogrel (600 mg). BMS-986120 demonstrated highly selective and reversible inhibition of PAR4 agonist peptide (100 μM)-stimulated P-selectin expression, platelet-monocyte aggregates, and platelet aggregation (P<0.001 for all). Compared with pretreatment, total thrombus area (μm^2/mm) at high shear was reduced by 29.2% (95% confidence interval, 18.3%–38.7%; P<0.001) at 2 hours and by 21.4% (9.3%–32.0%; P=0.002) at 24 hours. Reductions in thrombus formation were driven by a decrease in platelet-rich thrombus deposition: 34.8% (19.3%–47.3%; P<0.001) at 2 hours and 23.3% (5.1%–38.0%; P=0.016) at 24 hours. In contrast to aspirin alone, or in combination with clopidogrel, BMS-986120 had no effect on thrombus formation at low shear (P=nonsignificant). BMS-986120 administration was not associated with an increase in coagulation times or serious adverse events.

Conclusions—BMS-986120 is a highly selective and reversible oral PAR4 antagonist that substantially reduces platelet-rich thrombus formation under conditions of high shear stress. Our results suggest PAR4 antagonism has major potential as a therapeutic antiplatelet strategy.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT02439190.

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Key Words: aspirin ■ blood platelets ■ humans ■ monocytes ■ thrombosis

Platelets are central to thrombus formation, the leading cause of global mortality.[1] Antiplatelet drugs are of proven benefit for the treatment and prevention of atherothrombotic events in many clinical settings, but despite the introduction of newer agents in the last decade, important limitations persist. Aspirin and P2Y12 antagonists, the current standard of care oral antiplatelet agents in patients with acute coronary syndrome, stroke, and peripheral arterial disease, prevent thromboxane A2 and ADP platelet activation, respectively.[2–7] However, neither is effective against thrombin, the most potent of all platelet agonists,[8] and both are associated with an increased incidence of bleeding that restricts their use in sensitive populations (eg, elderly, cerebrovascular disease) and reduces their net clinical benefit.[5,9–12] For many patients, the residual risk of atherothrombotic events remains high,[5–7,10,13,14] and there is a clear need for novel agents that can provide equivalent (or superior) atherothrombotic efficacy with an improved safety profile.

In recent years, PAR4 (protease-activated receptor 4) antagonism has emerged as promising new antiplatelet strategy. PAR4 is a G-protein coupled receptor expressed on the platelet surface that together with PAR1 (protease-activated receptor 1) is responsible for thrombin-mediated platelet activation and aggregation.[15] Thrombin has a key role in the coagulation cascade, but by targeting the platelet receptor rather than the protease, this avoids directly interfering with thrombin-induced fibrin production. PAR1 has greater affinity for thrombin than PAR4, but despite early clinical promise,

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Nonstandard Abbreviations and Acronyms	
AP	agonist peptide
CI	confidence interval
ns	nonsignificant
PAR	protease-activated receptor
PI3K	phosphatidylinositol 3-kinase
PROBE	prospective randomized open-label blinded end point
TF	tissue factor

the addition of vorapaxar (the only licensed PAR1 antagonist) to standard care failed to meet its primary efficacy outcome in patients with acute coronary syndrome and was associated with an excess of major bleeding, especially intracranial hemorrhage, in phase3 clinical trials.[13,16] PAR4 was originally thought to simply provide redundancy to PAR1 platelet signaling at high thrombin concentrations.[17] However, because of differences in activation kinetics and downstream pathways, it is now evident that PAR1 and PAR4 have distinct and complementary roles in the early and late phases of platelet activation and aggregation, respectively.[18–20] PAR1 activation is brisk but transient and requires input from the P2Y12-PI3K (phosphatidylinositol 3-kinase) pathway to maintain platelet aggregation.[19,20] In contrast, PAR4 is activated at higher thrombin concentrations and induces a slow but prolonged intracellular signal that acts independently to sustain irreversible aggregation.[17,18,20] Furthermore, PAR4 activation occurs after ADP secretion, and thrombin depends on PAR4 but not PAR1 to induce full platelet spreading.[21] Thus, several lines of evidence indicate that although PAR1 and other agonist-signaling pathways may be more important for initiating platelet activation, the primary role of PAR4 appears to be in sustaining irreversible platelet aggregation and thrombus propagation. This suggests that PAR4 inhibition may protect against occlusive thrombus formation while avoiding interfering with hemostatic platelet responses to the same extent as PAR1 antagonists and other antiplatelet agents.[22]

BMS-986120 is a first-in-class, oral, highly selective, and reversible PAR4 antagonist antiplatelet agent. In preclinical animal models, BMS-986120 demonstrated potent antithrombotic activity with a substantially wider therapeutic window when compared with clopidogrel.[22] The purpose of the present phase 1 parallel-group PROBE trial (Prospective Randomized Open-Label Blinded End Point) was to build on these observations and examine for the first time, the antiplatelet and antithrombotic effects of BMS-986120 in humans using a translational model of ex vivo thrombosis. We determined whether reductions in thrombus formation were driven by a decrease in platelet-rich or fibrin-rich thrombus formation and whether these effects were greater under rheological conditions of low or high shear stress.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

All 40 volunteers (81 volunteers were screened) completed the study in full. The demographics and baseline characteristics of study volunteers were similar in the 2 treatment groups (Table). BMS-986120 was well tolerated with no clinically significant effect on any of the biochemical, hematologic, coagulation, physical, or ECG safety assessments conducted throughout the study (Table I in the online-only Data Supplement). There were no serious adverse events. One episode of minor bleeding was reported. This occurred 12 hours after aspirin administration, self-resolved, and did not recur.

Pharmacokinetic Profile of Oral BMS-986120

BMS-986120 was rapidly absorbed with peak plasma concentrations occurring at 2 hours (255±136 ng/mL; Figure 1). Plasma concentrations of BMS-986120 were halved by 4 hours (133±100 ng/mL) and <10% of the peak concentration by 24 hours (21±9 ng/mL).

Effect of BMS-986120 on Platelet Activation and Aggregation

BMS-986120 demonstrated strong and reversible inhibition of PAR4 agonist peptide (AP; 100 μM)-stimulated platelet activation and aggregation (P<0.001 for all). Compared with pretreatment, PAR4 AP-stimulated increases in platelet P-selectin expression (%), platelet-monocyte aggregates (%), and platelet aggregation (%) were reduced by 91.7% (95% confidence interval [CI], 81.0–102.4), 80.6% (95% CI, 68.6%–92.6%), and 85.0% (95% CI, 82.0–88.1) at 2 hours and by 53.9% (95% CI, 43.2%–64.7%), 41.1% (95% CI, 28.9%–53.2%), and 6.0% (95% CI, 2.9%–9.0%) at 24 hours (P<0.001 for all; Figure 2). Plasma concentrations of BMS-986120 correlated with P-selectin expression (ρ=−0.87), platelet-monocyte aggregates (ρ=−0.88), and platelet aggregation (ρ=−0.82; P<0.001 for all; Figure III in the online-only Data Supplement). There was no effect on PAR1 AP, ADP, or arachidonic acid platelet responses (P=nonsignificant [ns] for all; Figure 2).

Effect of Aspirin±Clopidogrel on Platelet Aggregation

Aspirin administration reduced arachidonic acid-stimulated platelet aggregation by 74.5% (95% CI, 71.6%–77.3%; P<0.001). In combination with clopidogrel, aspirin reduced arachidonic acid-stimulated platelet aggregation by 73.7% (95% CI, 70.9%–76.5%; P<0.001) and ADP-stimulated platelet aggregation by 41.9% (95% CI, 35.2%–48.7%; P<0.001), respectively (Figure IV in the online-only Data Supplement).

Effect of BMS-986120 on Ex Vivo Thrombus Formation

BMS-986120 reduced total thrombus formation at high shear (P<0.001) but not at low shear (P=ns; Figure 3). Compared with pretreatment, total thrombus area (μm²/mm) at high shear was reduced by 29.2% (95% CI, 18.3%–38.7%; P<0.001) at 2 hours and by 21.4% (95% CI, 9.3%–32.0%; P=0.002) at 24 hours. Plasma concentrations of BMS-986120 correlated with total thrombus formation at high shear (ρ=−0.47; P<0.001) but not at low shear (ρ=−0.18; P=ns; Figure III in the online-only Data Supplement).

Table. Baseline Characteristics of Study Volunteers

Test Variable	BMS-986120 (n=20)	Aspirin±Clopidogrel (n=20)
Men (%)	20 (100)	20 (100)
Age, y (SD)	23.6 (3.4)	28.7 (10.0)
BMI, kg/m^2 (SD)	23.6 (2.6)	25.4 (3.5)
Race (%)		
White	19 (95)	19 (95)
Black/African	1 (5)	0
Asian	0	1 (5)
Hemoglobin, g/dL (SD)	14.2 (0.42)	14.6 (0.85)
Platelet count, ×10^9 c/L (SD)	230 (45)	221 (49)
APTT, s (SD)	30.9 (2.2)	30.8 (2.6)
PT, s (SD)	12.3 (0.9)	11.9 (0.7)

APTT indicates activated partial thromboplastin time; BMI, body mass index; and PT, prothrombin time.

Reductions in total thrombus area were driven by a decrease in platelet deposition (Figure 4). At high shear, platelet-rich thrombus area was reduced by 34.8% (95% CI, 19.3%–47.3%; *P*<0.001) at 2 hours and 23.3% (95% CI, 5.1%–38.0%; *P*=0.016) at 24 hours. Reductions in fibrin-rich thrombus area at 2 (−14.7%; 95% CI, −22.5% to −6.2%; *P*=0.002) and 24 hours (−7.9%; 95% CI, −16.3% to 1.4%; *P*=0.09) were small by comparison. BMS-986120 had no effect on either platelet-rich or fibrin-rich thrombus formation at low shear (*P*=ns for all).

Effect of Aspirin±Clopidogrel on Ex Vivo Thrombus Formation

Aspirin and aspirin in combination with clopidogrel both reduced thrombus formation at high and low shear, also driven by decrease in platelet-rich thrombus. Aspirin reduced total thrombus area and platelet-rich thrombus area by 30.2% (95% CI, 15.6%–42.2%; *P*<0.001) and 41.7% (95% CI, 22.9%–56.0%; *P*<0.001), respectively, and by 32.4% (95% CI, 18.3%–44.0%; *P*<0.001) and 46.4% (95% CI, 29.1%–59.5%; *P*<0.001), respectively, when used in combination with clopidogrel.

In contrast to BMS-986120, aspirin and aspirin in combination with clopidogrel both reduced total thrombus area at low shear (−17.4%; 95% CI, −27.0% to −6.5%; *P*=0.003 and −13.5%; 95% CI, −23.6% to −2.1%; *P*=0.02). There was no effect on fibrin-rich thrombus deposition at low or high shear (*P*=ns for all).

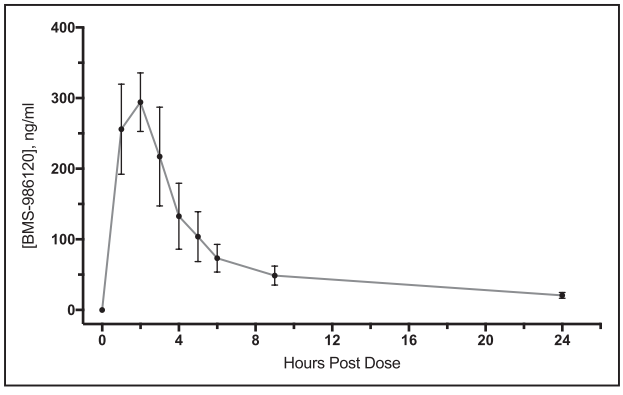

Figure 1. Pharmacokinetics of BMS-986120. BMS-986120 was rapidly absorbed with a half-life of 4 h. Data shown are mean plasma concentrations of BMS-986120 (±95% confidence intervals) after administration of a single oral 60-mg dose.

Discussion

In this phase 1 PROBE designed clinical trial, we have shown for the first time that PAR4 antagonism with BMS-986120 reduces ex vivo human thrombus formation under conditions representative of deep arterial injury in a stenosed coronary artery. BMS-986120 demonstrated selective and reversible antiplatelet effects with concentration-dependent inhibition of thrombus formation and PAR4 AP-stimulated platelet activation and aggregation. Our results provide further insights into the role of PAR4 in human thrombogenesis and raise major promise for BMS-986120 as an antiplatelet agent in the treatment and prevention of arterial thrombosis.

Assessment of the antiplatelet and antithrombotic effects of PAR4 inhibition has previously been limited by a lack of compound specificity and availability.[23–25] In comparison with earlier compounds, including P4pal-10, YD-3, and its derivative ML354,[25–27] BMS-986120 has antiplatelet activity against α thrombin, demonstrated greater potency and selectivity of effect in preclinical and phase 1 studies of platelet inhibition, and is the first orally bioavailable PAR4 antagonist.[22,28] In the present study, a single dose of BMS-986120 resulted in near complete inhibition of PAR4 AP-stimulated platelet activation and aggregation at 2 hours, with a return toward baseline at 24 hours. Importantly, there was no effect on PAR1 AP, ADP, or arachidonic acid-stimulated platelet activity. Our data, therefore, add to previous studies indicating that BMS-986120 is a highly selective and reversible antiplatelet agent with potent activity against PAR4-stimulated platelet activation and aggregation in humans.

The antithrombotic effects of BMS-986120 in humans were examined using the Badimon perfusion chamber—a well validated model for measuring ex vivo thrombus formation in humans.[29–36] Using the same model and under the same flow conditions, previous studies in healthy volunteers have demonstrated reductions in high shear thrombus formation of 18.7% after a single 300-mg oral dose of clopidogrel, 28% with a 60-mg oral dose of edoxaban and 56% with extracorporeal coadministration of tirofiban (50 ng/mL).[29,36,37] In the present study, a single dose of BMS-986120 (60 mg) reduced high shear thrombus formation by nearly a third. This is consistent with preclinical animal data[22,23] and comparable with reductions in thrombus formation we observed with high loading doses of aspirin and clopidogrel. Importantly, therefore, we have shown that oral PAR4 antagonism with BMS-986120 substantially reduces ex vivo human thrombus formation. Moreover, reductions were similar in magnitude to clinically approved antiplatelet agents suggesting a high probability of in vivo antithrombotic efficacy.

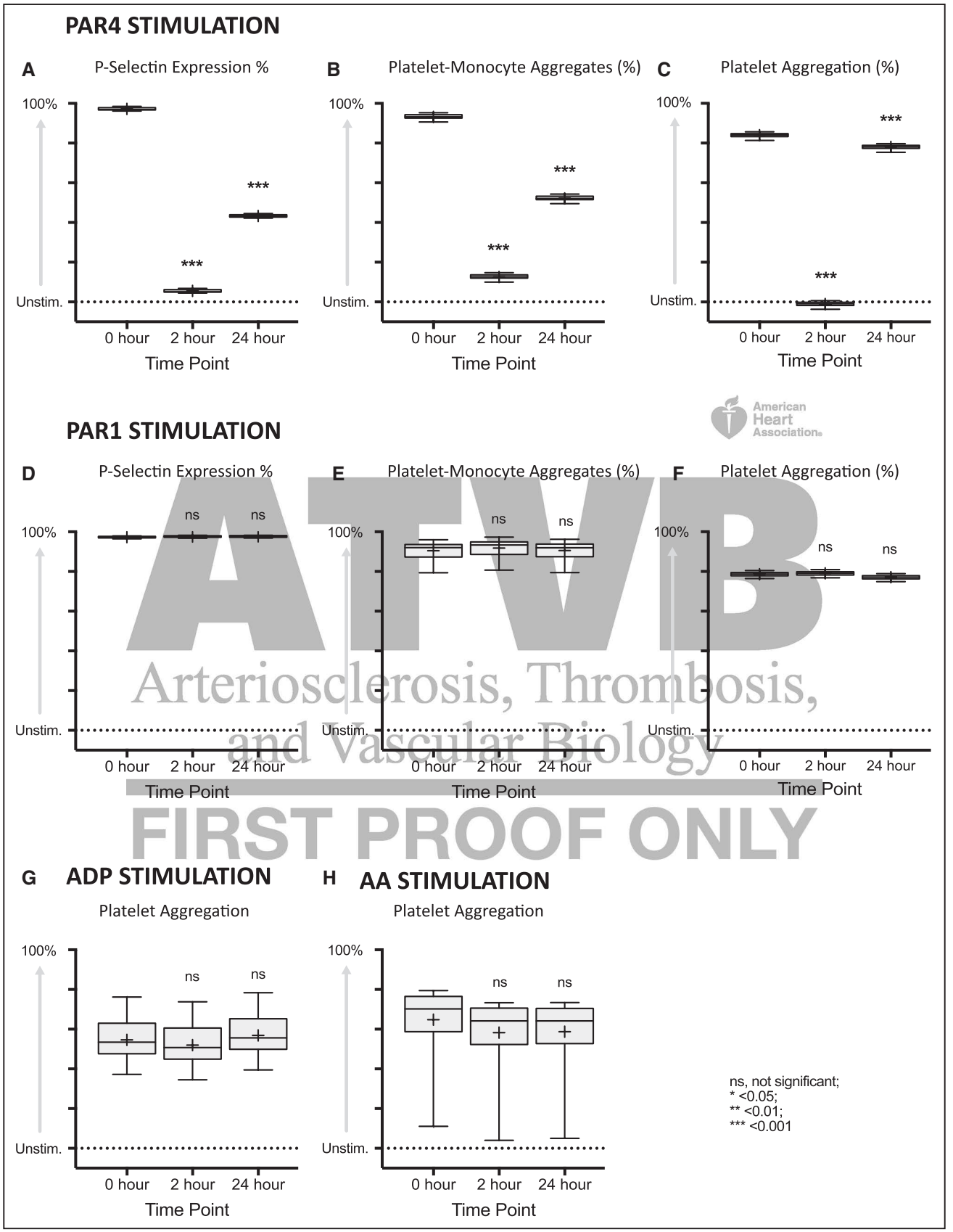

Figure 2. BMS-986120 demonstrated highly selective, potent, and reversible inhibition of PAR (protease-activated receptor) 4-stimulated platelet activation and aggregation. Box plots of platelet activation and aggregation in response to (**A**–**C**) PAR4 Agonist peptide (AP; 100 μM), (**D** and **E**) PAR1 AP (100 μM), (**F**) PAR1 AP (25 μM), (**G**) ADP (10 μM), and (**H**) arachidonic acid (AA; 5 mmol/L), in volunteers (*Continued*)

Figure 2 Continued. randomized to BMS-986120. Data shown are the adjusted mean (+) normalized to unstimulated values. The line within the box represents the median, upper and lower edges of the box represent the 75th and 25th percentiles, and upper and lower whiskers represent the 95th and 5th percentiles. Statistical comparisons (least significance difference test) vs 0 h are represented above each plot. ns indicates nonsignificant. *P<0.05, **P<0.01, ***P<0.001.

BMS-986120 seemed to have less of an effect on thrombus formation at low shear than either aspirin alone or aspirin in combination with clopidogrel. Although further studies are required to confirm whether PAR4 antagonism is more selective for inhibiting thrombus formation at high shear than existing agents, distinct mechanisms of platelet aggregation are known to operate under different rheological conditions.[38,39] Low shear rates reflect flow conditions found in patent epicardial arteries and some veins, whereas the majority of atherothrombotic events invariably occur at areas of high shear stress seen in diseased arteries.[40,41] Indeed, most myocardial infarctions arise from stenotic atherosclerotic plaques with rheological conditions comparable with those in our high shear chamber.[42–44] Antiplatelet agents that are more selective for inhibiting thrombus formation at high shear may allow at-risk vascular beds to be targeted with greater specificity. Given many treatment-related bleeding events are likely to occur from vessels with low shear rates,[45–49] this could facilitate a wider safety profile.

As expected from an antiplatelet agent, reductions in thrombus were driven by a decrease in platelet deposition; however, there was also a small but significant reduction in fibrin-rich thrombus formation. PAR4 is reported to be the predominant platelet PAR responsible for phosphatidylserine exposure, microparticle shedding, and thrombin generation.[50] Our results add to these studies, indicating that PAR4 may have a role in platelet procoagulant activity during ex vivo human thrombus formation. Whether this is beneficial or not is uncertain, but it is worth noting BMS-986120 was not associated with an increase in coagulation assay times, and no bleeding-related clinical findings or adverse events were reported in a previous phase 1 single- and multiple-ascending dose study.[28]

PAR4 is expressed within the vasculature, and PAR4 antagonism may, in addition to protecting against thrombosis, serve to limit vascular complications in at-risk patients. Human vascular smooth muscle cells upregulate PAR4 in response to glucose,[51] and elevated expression of

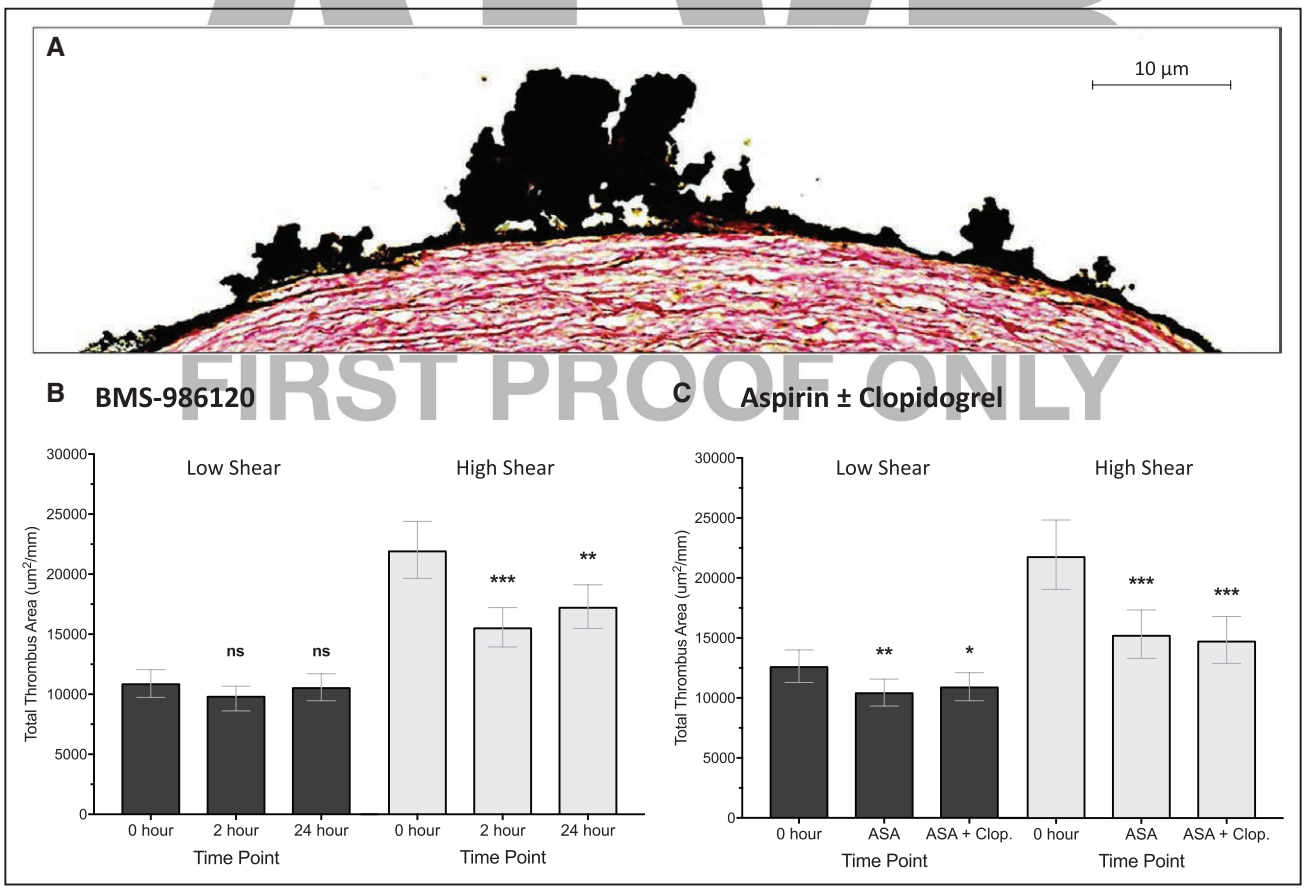

Figure 3. BMS-986120 reduced thrombus formation at high shear but not at low shear. **A**, Representative image of porcine aortic media exposed to human blood at high shear stained to quantify total thrombus area. Sections were stained with polyclonal goat antihuman fibrin(ogen) antibody and CD61 monoclonal mouse antihuman antibody before treatment with 3,3′-diaminobenzidine substrate chromogen. Sections were then counterstained with a modified Masson trichrome (hematoxylin and sirius red, 0.1%). Effect of (**B**) BMS-986120 and (**C**) aspirin (ASA)±clopidogrel (Clop.) on total thrombus area at high and low shear. Statistical comparisons (least significance difference test) vs 0 h are represented above each plot. ns indicates nonsignificant. *P<0.05, **P<0.01, ***P<0.001.

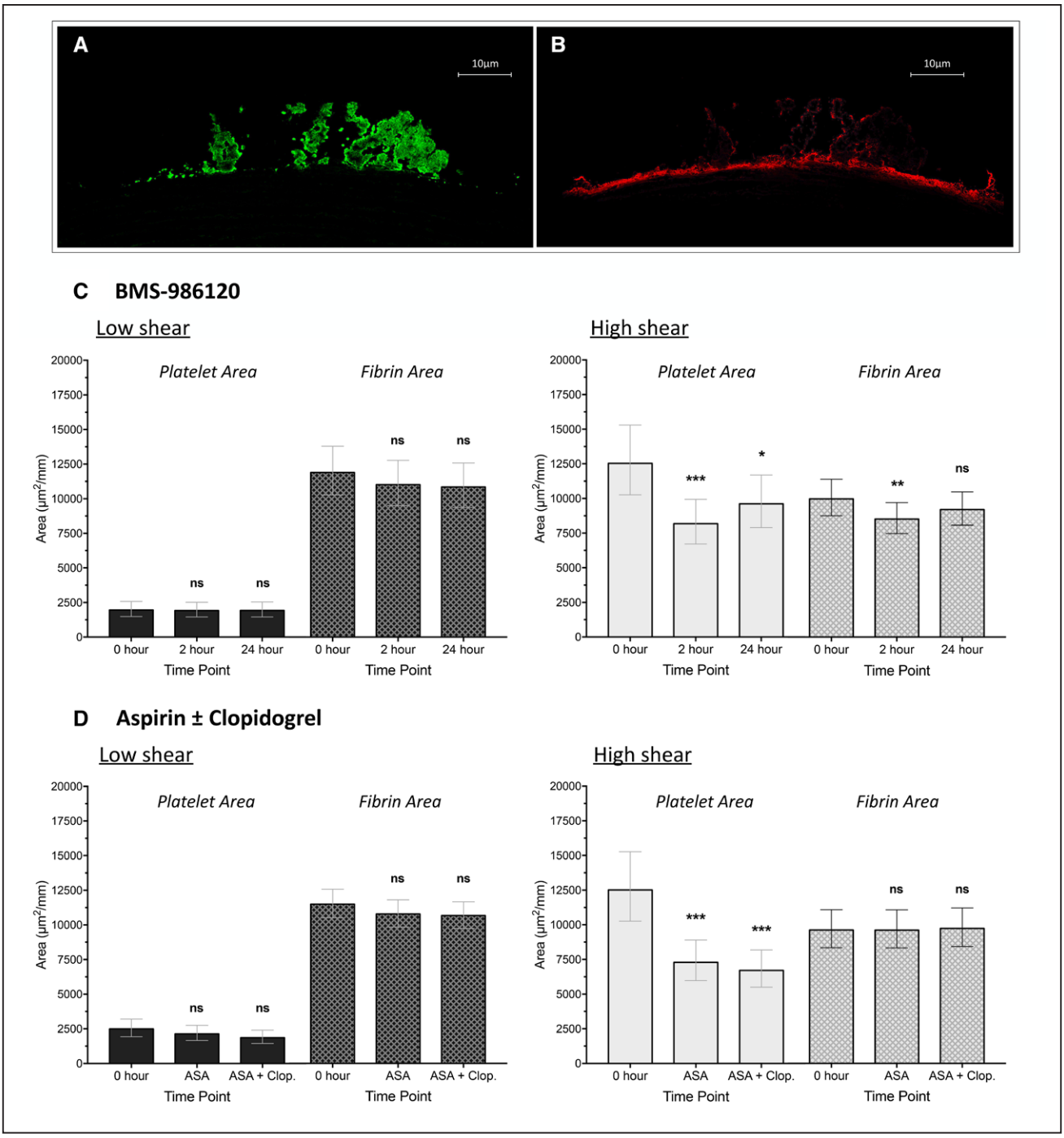

Figure 4. Reductions in thrombus formation were driven by a decrease in platelet-rich thrombus formation. Representative image of thrombus formed at high shear stained to allow quantification of (**A**) platelet-rich and (**B**) fibrin-rich thrombus area. Sections were stained with polyclonal goat antihuman fibrin(ogen) antibody and CD61 monoclonal mouse antihuman antibody before counterstaining with tyramide Cy3 and FITC. Effect of (**C**) BMS-986120 and (**D**) aspirin (ASA)±clopidogrel (Clop.) on platelet and fibrin deposition at low and high shear. Data shown are adjusted means±95% confidence intervals. Statistical comparisons (least significance difference test) vs 0 h are represented above each plot. ns indicates nonsignificant. *P<0.05, **P<0.01, ***P<0.001.

PAR4 has been reported in the tunica media of atherectomy and saphenous vein tissue from patients with diabetes mellitus.[51] Moreover, PAR4 deficiency protected against excessive remodeling induced by carotid artery ligation in streptozotocin-diabetic mice.[52] PAR4, therefore, seems to be a key regulator of exaggerated intimal thickening in diabetes mellitus, and future studies examining the antiproliferative potential of PAR4 antagonism would be of significant therapeutic interest.

Our study has some limitations. First, although the exposed porcine aortic media presents many of the common constituents of a disrupted atherosclerotic plaque, including type I collagen, it may not contain tissue factor (TF).[53–55] TF activates the coagulation cascade and is an important

contributor to thrombogenicity.[56,57] Nevertheless, this does not overly limit our model for the assessment of thrombosis because binding of blood-borne circulating TF is sufficient to allow activation of the coagulation cascade and thrombus propagation.[53,54,58–60] Indeed, previous studies have demonstrated that thrombus formed from human blood perfused over exposed porcine tunica media (devoid of TF) stains heavily for TF.[53,54] Second, we assessed a single oral dose of BMS-986120 and did not explore the effect of prolonged BMS-986120 administration on thrombus formation, such as would occur with the secondary prevention of myocardial infarction and stroke. However, because this was the phase 1 trial designed to examine the antithrombotic effects of oral PAR4 antagonism in humans for the first time, we felt our study design was appropriate. Third, BMS-986120 was dosed in isolation, and future studies to determine the antiplatelet and antithrombotic effects of PAR4 antagonism in combination with current agents would be of interest. Finally, although no episodes of bleeding occurred in volunteers administered BMS-986120 and BMS-986120 was not associated with an increase in bleeding times in a previous phase 1 safety and tolerability study,[28] the safety profile of PAR4 antagonism in humans remains to be defined.

In conclusion, we have demonstrated that PAR4 antagonism with BMS-986120—a highly selective and reversible oral PAR4 antagonist—substantially reduces ex vivo thrombus formation in healthy volunteers under conditions of high shear stress. BMS-986120 was well tolerated with no change in coagulation assays or serious adverse events. Given the potential hemostatic sparing effects of PAR4 antagonism, our results suggest that BMS-986120 has major potential as a novel antiplatelet agent and that further investigation in clinical trials is warranted.

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Disclosures

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Highlights

- Inhibition of thrombin-mediated platelet activation through PAR4 (protease-activated receptor 4) antagonism represents a promising new antiplatelet strategy because of the potential for reduced bleeding.
- BMS-986120 is a first-in-class, oral, highly selective, and reversible PAR4 antagonist antiplatelet agent.
- A single dose of BMS-986120 substantially reduced ex vivo thrombus formation in healthy volunteers under conditions of high shear stress, driven by a reduction in platelet-rich thrombus deposition.
- Our results suggest PAR4 antagonism with BMS-986120 holds major promise as a novel antiplatelet strategy because of the potential for a wider therapeutic window in terms of antithrombotic efficacy and bleeding risk.

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PAR4 (Protease-Activated Receptor 4) Antagonism With BMS-986120 Inhibits Human Ex Vivo Thrombus Formation

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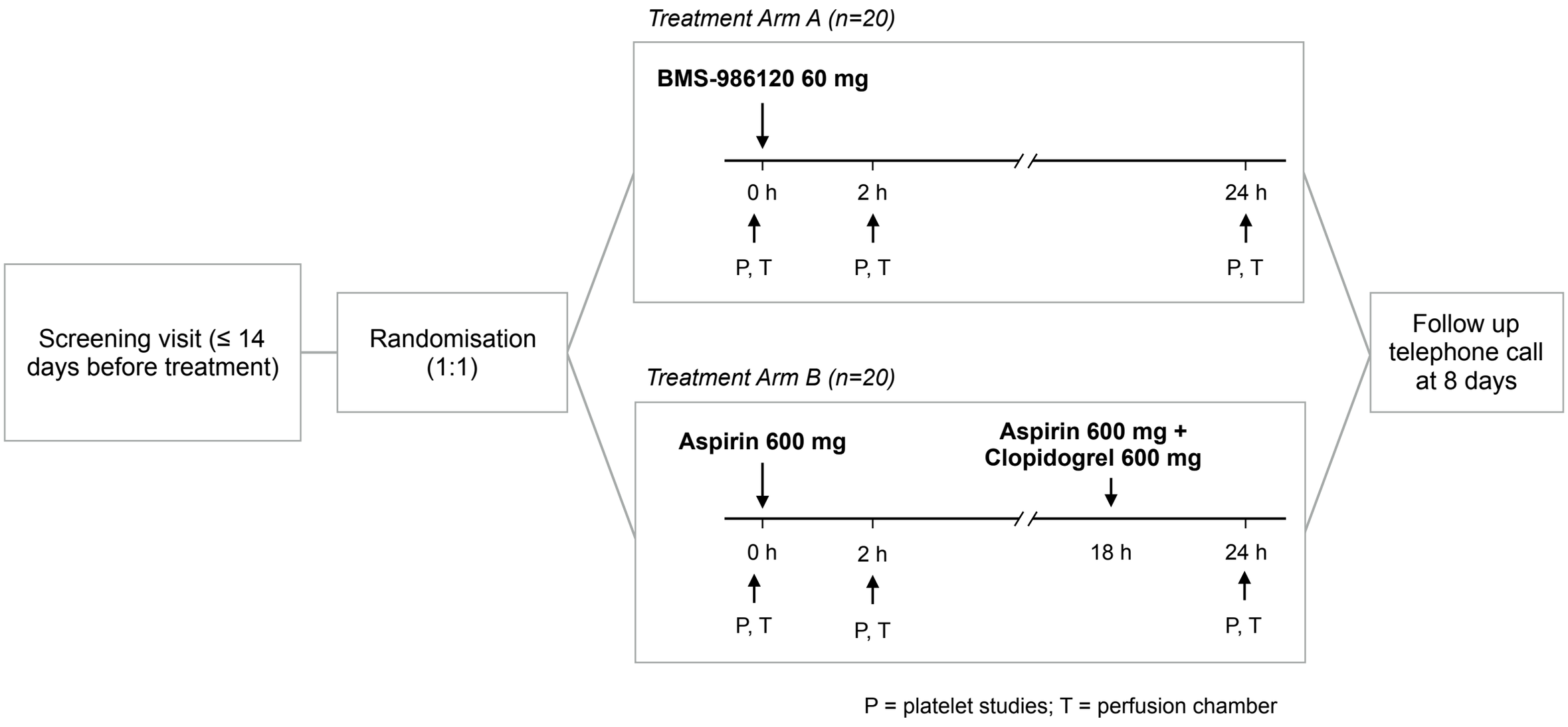
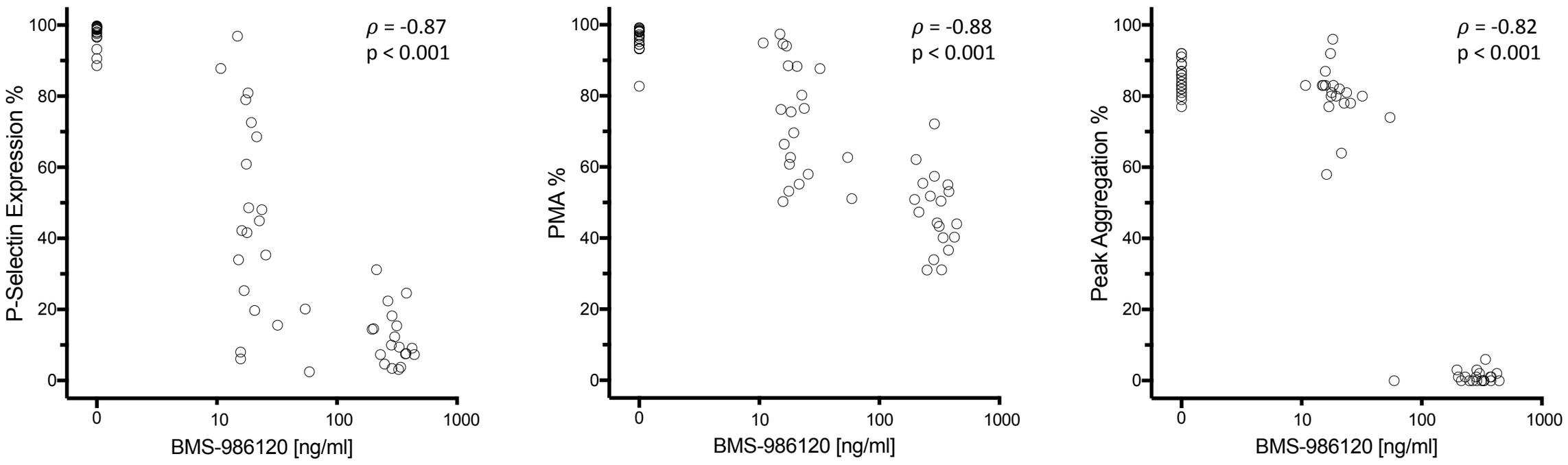


Figure I. Schematic overview of study design.

Table I. Safety Assessments

Test variable	BMS-986120 (n=20)			Aspirin ± Clopidogrel (n=20)		
	0 hour	*2 hour*	*24 hour*	*0 hour*	*ASA*	*ASA + Clop*
ALT, U/L (SD)	19.0 (6.1)	18.7 (6.2)	17.8 (6.4)**	26.3 (14.8)	25.9 (14.2)	25.4 (13.3)
AST, U/L (SD)	20.7 (4.6)	20.9 (5.7)	18.0 (3.6)***	23.6 (7.4)	23.4 (6.8)	21.0 (4.5)**
Bilirubin, mg/dL (SD)	0.70 (0.3)	0.80 (0.3)*	0.62 (0.3)	0.76 (0.2)	0.73 (0.2)	0.66 (0.2)**
Creatine Kinase, U/L (SD)	197 (190)	187 (188)	115 (83)***	186 (144)	171 (128)	108 (61)***
Urea, mmol/L (SD)	5.1 (0.96)	4.6 (0.76)**	4.1 (0.77)***	5.0 (1.0)	4.7 (0.93)	4.5 (0.98)**
Creatinine, mg/dL (SD)	0.83 (0.06)	0.81 (0.09)	0.82 (0.08)	0.89 (0.11)	0.86 (0.09)	0.86 (0.10)
Haemoglobin, g/dL (SD)	14.2 (0.4)	14.2 (0.4)	14.2 (0.3)	14.6 (0.8)	14.4 (0.8)	14.3 (1.3)
Platelet count, $x10^9$ c/L (SD)	229 (45)	227 (46)	228 (46)	221 (49)	220 (50)	216 (53)
APTT, seconds (SD)	30.9 (2.1)	30.3 (2.7)	30.4 (2.1)	30.8 (2.5)	30.3 (3.3)	30.1 (2.9)
PT, seconds (SD)	12.3 (0.9)	12.3 (0.9)	12.1 (0.8)	11.9 (0.7)	12.3 (0.7)**	12.1 (0.7)
QTCf interval, milliseconds (SD)	405 (11.8)	411 (17.9)	405 (15.9)	401 (16.3)	402 (13.4)	399 (10.8)

Data shown are the adjusted means with standard deviation. All significant differences (Least Significance Difference test) versus 0 hour are presented: * p<0.05, ** p<0.01, *** p<0.001. Abbreviations used: ASA, aspirin; Clo, clopidogrel. ALT, alanine transaminase; ASA, Aspirin; AST, aspartate transaminase; APTT, activated partial thromboplastin time; Clop, Clopidogrel; PT, prothrombin time; QTCf, QTC interval corrected for heart rate by Fridericia's formula; and SD, standard deviation



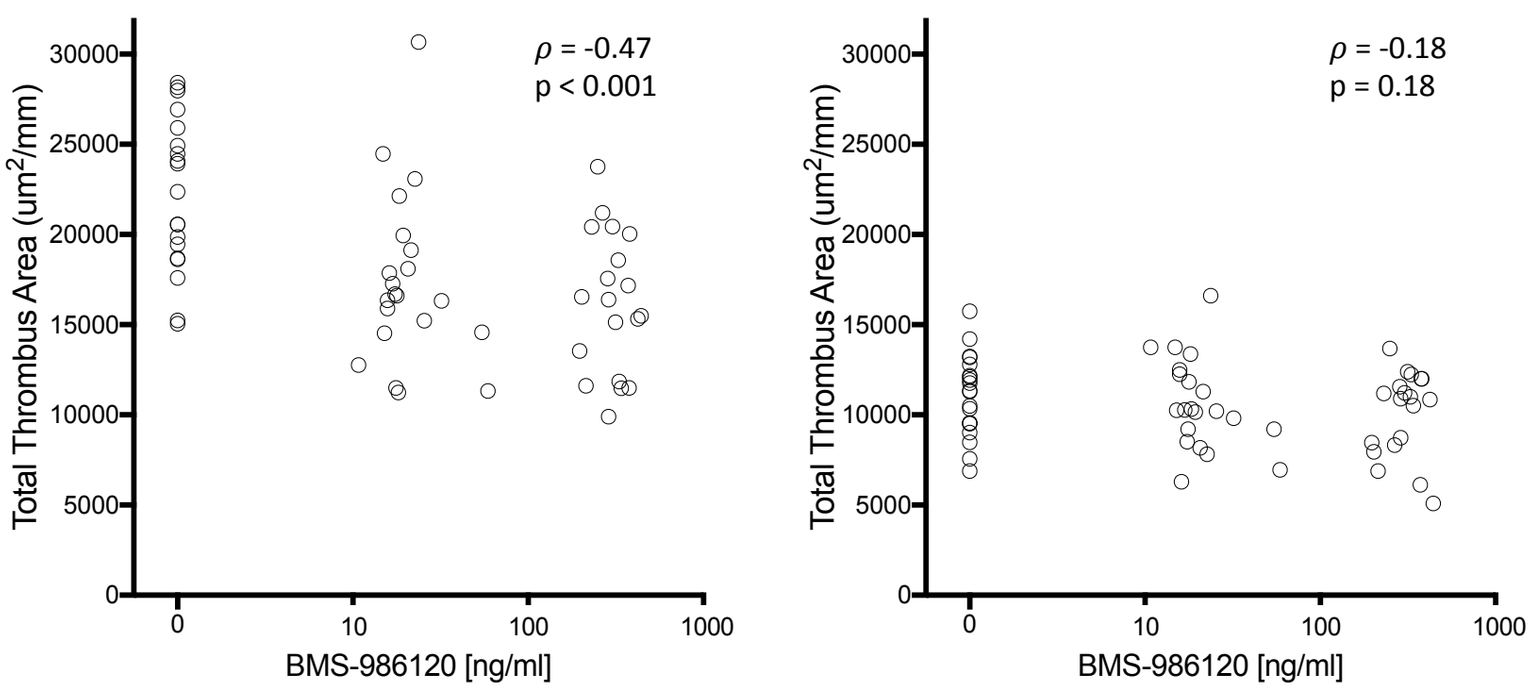


Figure II. Correlations between plasma concentrations of BMS-986120 and selected study endpoints.

Data shown are scatter plots of [A] PAR4 AP stimulated p-selectin expression, [B] PAR4 AP stimulated platelet-monocyte aggregates, [C] PAR4 AP stimulated platelet aggregation, [D] total thrombus area at high shear, and [E] total thrombus at low shear in volunteers randomised to BMS-986120. Correlation coefficients (ρ) and p-values were determined by Spearman's rank-order correlation.

Platelet Aggregation

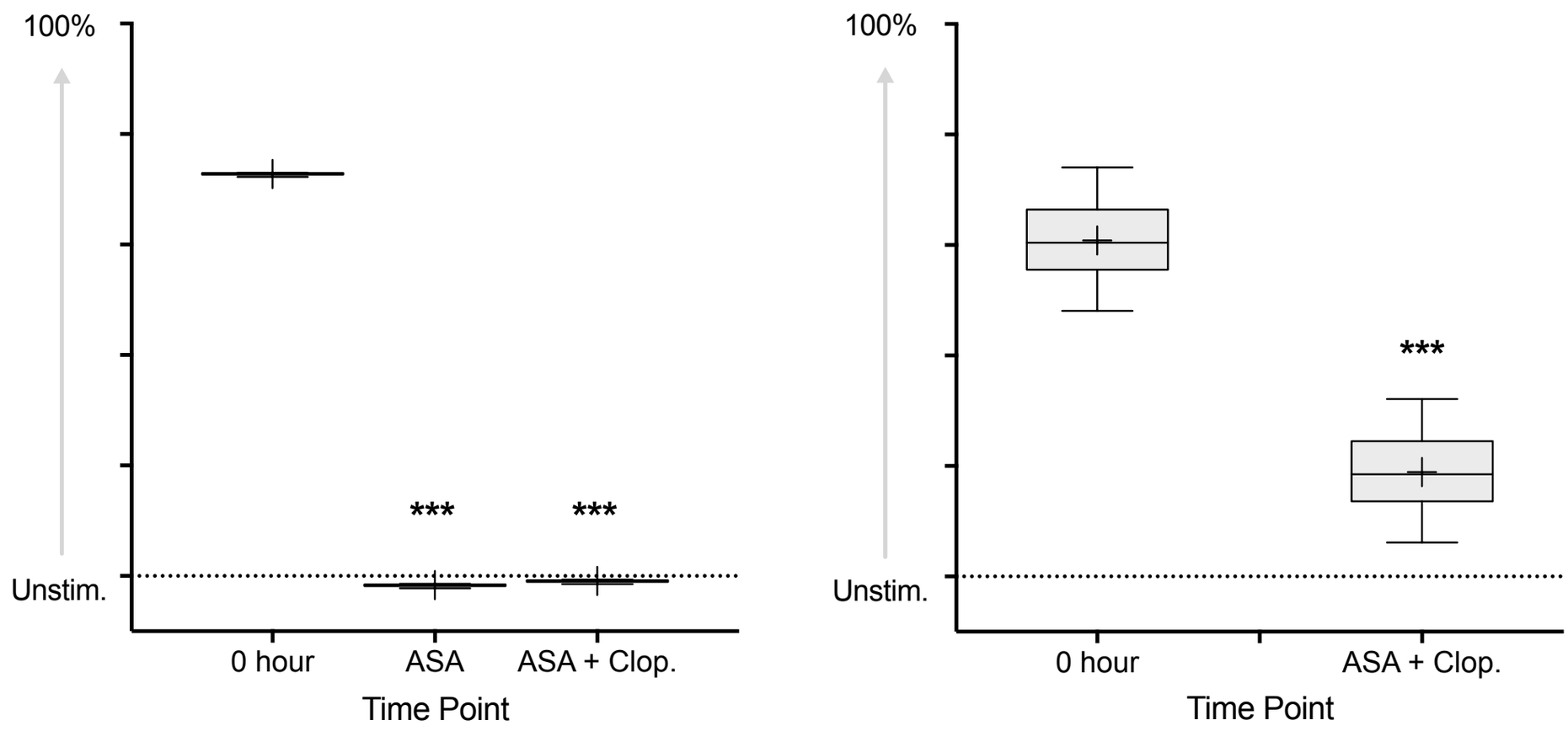


Figure III. Box plots of platelet aggregation in response to (A) arachidonic acid [5 mM] and (B) adenosine diphosphate [10 µM] in volunteers randomised to aspirin ± clopidogrel.

Data shown are the adjusted means (+) normalised to unstimulated values. The line within the box represents the median, upper and lower edges of the box represent the 75th and 25th percentiles, and upper and lower whiskers represent the 95th and 5th percentiles. Statistical comparisons (Least Significance Difference test) versus 0 hour are shown above each plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

SUPPLEMENTAL MATERIAL

Methods

Study Design

This was a phase I parallel group (n=20 per treatment arm) prospective randomized open-label blinded endpoint (PROBE) trial conducted at a single site (Clinical Research Facility, Royal Infirmary of Edinburgh, Scotland) between the 23rd September 2015 and 1st March 2016. *Ex vivo* platelet aggregation, platelet activation and thrombus formation were measured at 0 (pre-treatment), 2 and 24 h after oral administration of (a) 60 mg of BMS-986120 or (b) 600 mg aspirin with a second 600 mg aspirin and 600 mg clopidogrel at 18 h (Figure I in the online-only Data Supplement). Aspirin ± clopidogrel were included as a positive control and assay validation tool.

The trial was sponsored by Bristol-Myers Squibb (BMS) and was designed collaboratively with the host academic center. The study was approved by the local research ethics committee, conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Clinical Trial Authorization was provided by the Medicines and Healthcare products Regulatory Authority (MHRA) of the United Kingdom.

Study End-Points

The primary outcome was the effect of BMS-986120 on total thrombus area as compared to pre-treatment. Secondary and exploratory end-points included the effect of study drug (BMS-986120 or aspirin ± clopidogrel) on platelet aggregation, p-selectin expression, platelet-monocyte aggregates, and thrombus composition (platelet- and fibrin-rich thrombus area).

Study Population

Healthy non-smoking male and female volunteers between the ages of 18 and 65 years (inclusive) and with a body mass index (BMI) of 18 to 32 kg/m^2 underwent screening including detailed medical history, physical examination, laboratory blood tests, urinalysis and 12-lead electrocardiogram (ECG). Exclusion criteria were women of child-bearing potential and any clinically significant coexisting condition including hypertension, hyperlipidemia, diabetes mellitus, gastrointestinal disease that could affect drug absorption, coagulopathy, recent infective or inflammatory condition, known liver disease or screening blood tests indicative of renal, liver, clotting, thyroid or hematological abnormality. Volunteers must not have been taking any prescription medications for 4 weeks, over-the-counter medications, herbal supplements and vitamins for 1 week, and alcohol or caffeine containing products for 72 hours prior to and for the duration of the study.

Dose Selection

BMS-986120 is a competitive, reversible inhibitor of PAR4 AP induced platelet aggregation (K_{on}=0.12 ± 0.043 $nM^{-1}min^{-1}$, K_{off}=0.0082 ± 0.0016 min^{-1}, K_d=0.098 ± 0.016 nM). In cynomolgus monkeys, BMS-986120 demonstrated dose-dependent (0.2-1.0 mg/kg) preservation of carotid arterial flow following

electrolytic injury at the expense of a slight increase in mesenteric and kidney bleeding times[1]. In a single ascending (0.5-180 mg) and multiple ascending dose study (2.5-100 mg daily for up to 14 days) in healthy volunteers, BMS-986120 was found to be safe and well tolerated with complete and reversible inhibition of PAR4 agonist peptide (AP) stimulated platelet aggregation at ≥10 mg daily[2]. On the basis of these studies, a 60 mg dose was selected for the present phase 1 trial as this was calculated to be sufficient to inhibit platelet aggregation 2 h post dose and would be at the edge of a potential pharmacodynamic effect at 24 h. This would allow for "dose ranging" with a single dose of BMS-986120 whilst remaining well within the safety experience.

Doses of aspirin (600 mg) and clopidogrel (600 mg) were selected to reflect the maximal antithrombotic efficacy that might reasonably be expected in clinical practice following initiation of these antiplatelet agents in an acute setting.

Study Outcome Measures

Blood Sampling and Agonists

All blood samples for pharmacodynamic and pharmacokinetic assessments were drawn uncuffed through a 17G cannula in the ante-cubital fossa. For each time point, the first 2.5 mL of blood was discarded. PAR1 and PAR4 APs (SFLLRN and A-Phe(4-F)-PGWLVKNG respectively) were provided by Bristol-Myers Squibb (Princeton, USA), adenosine diphosphate (ADP) by Sigma-Aldrich (Gillingham, UK) and arachidonic acid (AA) by Alpha Laboratories (Eastleigh, UK).

Pharmacokinetic Assessment

Plasma concentrations of BMS-986120 were determined at 0, 1, 2, 3, 4, 5, 6, 9 and 24 h post treatment using a validated liquid chromatography tandem-mass spectrometry (LC-MS/MS) method with a lower limit of quantification (LLQ) of 0.250 ng/mL, with an accuracy coefficient of variation of <5 % and precision (intra- and inter-assay) coefficients of variation of <10 %. Blood samples were collected into 3 mL K_2EDTA vacutainers (Becton-Dickinson, Cowley, UK) and placed on wet ice. Within 1 h of collection, samples were centrifuged at 1200 *g* (2-8 °C) for 10 min. Plasma was decanted and stored at -20 °C before analysis.

Platelet Aggregation

Platelet aggregation was assessed by optical aggregometry (PAP-8E; Bio/Data Corp, Horsham, PA, USA) of platelet-rich plasma (PRP). To obtain PRP, 18 mL of blood was collected, mixed immediately with 2 mL of 3.8 % sodium citrate, and then centrifuged at 300 *g* (room temperature) for 15 min. For reference, 2 mL of PRP was centrifuged at 5500 *g* for 6 min to obtain platelet-poor plasma (PPP). All samples were allowed to equilibrate for 10 min (37 °C) after the addition of agonist and the peak aggregation recorded.

Platelet Activation

Platelet p-selectin expression and platelet-monocyte aggregates were determined by flow cytometry. Blood (5 mL) was collected into 50 μL of 75

mM D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK; Enzo Life Sciences, Exeter, UK) then immediately aliquoted into eppendorfs pre-filled with or without agonist and the following conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD14, phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-conjugated CD42a (Becton-Dickinson). All antibodies were diluted 1:10. Samples were incubated for 20 min at room temperature before fixing with 1 % paraformaldehyde (p-selectin) or FACS-Lyse (Becton-Dickinson; platelet-monocyte aggregates). All samples were analysed within 24 h using a FACSCalibur flow cytometer (Becton-Dickinson). Data analysis was performed using FlowJo v10 (Treestar, Oregon, USA).

Ex Vivo Perfusion Model of Thrombosis

The effect of study compound on ex vivo thrombus formation was assessed using the Badimon perfusion chamber as previously described[3]. In brief, a pump was used to draw native (unanticoagulated) blood directly from an antecubital vein through a series of three cylindrical perfusion chambers maintained at 37°C in a water bath. Each chamber contained a strip of porcine aorta from which the intima and a thin layer of media had been removed. The ultrastructure of porcine aorta closely resembles that of human arteries and by removing the intima and a thin layer of media, blood is exposed to collagen fibres (type I and type III), proteoglycans, basement membrane, elastin, smooth muscle cells and other constituents common to an atherosclerotic plaque[4-8]. Rheological conditions in the first chamber were set to simulate those of patent medium-sized coronary arteries (inner lumen diameter, 2.0 mm; vessel wall shear rate, 212 s^{-1}; mean blood velocity, 5.3 cm/s; Reynolds number: 30), whereas those in the second and third chambers simulate those of mild to moderately stenosed coronary arteries (inner lumen diameter, 1.0 mm; vessel wall shear rate: 1690 s^{-1}; mean blood velocity, 21.2 cm/s; Reynolds number: 60). Shear conditions at the vessel wall were calculated from the expression for shear rate given for a Newtonian fluid in tube flow[9,10]. Each study lasted for exactly 5 min during which flow was maintained at a constant rate of 10 mL/min. All studies were performed using the same perfusion chamber and by the same operator.

Histomorphometric Analysis

As thrombus forms along the entire length of the exposed porcine aortic strip, the mean transverse cross-sectional area gives a reliable reflection of total thrombus[6]. Following fixation, the proximal and distal 1 mm of the exposed substrate were discarded and the remainder cut into eight segments. Individual segments were then embedded in paraffin wax from which 4-μm sections were prepared for histomorphometric analysis.

To detect total thrombus area, endogenous hydrogen peroxide activity was blocked using 3 % hydrogen peroxide solution (Leica Microsystems GmbH, Wetzlar, Germany) for 5 minutes. Sections were then incubated at room temperature for 1 hour with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/mL, Dako, Glostrup, Denmark; Cat. No. A0080) and monoclonal mouse anti-human CD61 antibody (1.28 μg/ml, Dako; Cat. No. M0753). Antigen visualisation was performed using a Bond Polymer refine detection kit

(Leica Microsystems GmbH) and treatment with 3,3'-diaminobenzidine substrate chromogen (66 mM, Dako). Finally, sections were counterstained with a modified Masson's trichrome (hematoxylin and sirius red 0.1 %; Figure II in the online-only Data Supplement).

To examine the effect of study drug(s) on fibrin-rich and platelet-rich thrombus formation, endogenous hydrogen peroxide activity was blocked using 3 % hydrogen peroxide solution (VWR, Radnor, PA, USA) for 10 min and non-specific binding blocked using 20 % normal goat serum (Biosera, Nuaille, France) in Tris-Buffered Saline with 0.01% Tween (TBST)). Sections were then incubated with polyclonal rabbit anti-human fibrin(ogen) antibody (1.2 μg/ml) to detect fibrin and CD61 monoclonal mouse anti-human antibody (0.32 μg/ml) to detect platelets. Following TBST washes, goat anti-rabbit peroxidase (1:500; Abcam, Cambridge, UK) was applied and the presence of antigen visualized with Tyramide Cy3 (1:50; Perkin Elmer, Boston, MA, USA; Cat. no. NEL744B001KT) and FITC (1:50; Perkin Elmer, Waltham, MA, USA; Cat. no. NEL741B001KT) before nuclear counterstaining with DAPI (5 μg/ml; Sigma-Aldrich; Cat. No. D9542).

Prior to the first experimental sample, non-specific binding of the primary antibodies was excluded using tissue negative controls (perfusion chamber porcine sections exposed to saline rather than blood). To ensure staining for platelets and fibrin(ogen) antigen was the result of detection of the antigen, secondary antibody controls (with the primary antibody absent) were run in parallel for each volunteer. No labelling was observed.

A semi-automated slide scanner (Axioscan Z1; Zeiss, Jena, Germany) and image analysis software (Definiens, Munich, Germany) were used by a blinded operator to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers based on colour were established to detect total thrombus, platelet and fibrin area.

Safety and tolerability

The primary safety end-point was the incidence of serious adverse events (SAEs) or death during and for up to 30 days post dosing. Adverse events (AEs) not meeting the SAE threshold were also recorded. All volunteers received telephone follow up on day 8. Reports of SAEs and AEs could originate from the volunteer, investigator or study personnel. Additional safety endpoints included changes in hematological and biochemical indices, hematuria (including microhematuria), alteration in the 12-lead electrocardiogram (ECG), or abnormal findings on physical examination performed at baseline, 2 and 24 h post dosing.

Statistical Analysis

Following study completion, the database was locked and all statistical analyses carried out independent of the sponsor. The demographic and baseline characteristics of volunteers are expressed as mean ± standard deviation (SD) for continuous variables and percentages for categorical variables. The effect of study drug(s) on endpoints was assessed by general

linear mixed effects models, with perfusion procedure (baseline, 2 and 24 h) as fixed effects and subjects as random effects. Mean within-subject differences for the change from baseline were generated and analysed using the Least Significance Difference (LSD) test. Prior to model fitting, total thrombus area, platelet area and fibrin area were log-transformed. Associations between plasma concentrations of BMS-986120 and study end-points were determined by Spearman's rank-order correlation (ρ). Two sided p-values of ≤0.05 were considered statistically significant. Analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, New York) and R version 3.3.1 (R Project for Statistical Computing, Vienna, Austria).

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